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Identification, virulence, and mass spectrometry of toxic ECP fractions of West Alabama isolates of *Aeromonas hydrophila* obtained from a 2010 disease outbreak

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ABSTRACT

In West Alabama, disease outbreaks in 2009 caused by *Aeromonas hydrophila* have led to an estimated loss of more than \$3 million. In 2010, disease outbreak occurred again in West Alabama, causing losses of hundreds of thousands of pounds of market size channel catfish. During the 2010 disease outbreak in West Alabama, four isolates of *A. hydrophila* were cultured from the kidney tissues of diseased channel catfish. Both analytical profile index (API) 20 E biochemical tests and 16S–23S rRNA sequencing results confirmed the four isolates as *A. hydrophila*. Virulence studies revealed that the four isolates were highly virulent to channel catfish by intraperitoneal injection, with LD_{50} value of $\sim 1.3 \times 10^5$ CFU/fish. Extracellular proteins (ECPs) of *A. hydrophila* are well known to be toxic to fish. Therefore, ECPs of the four 2010 West Alabama isolates of *A. hydrophila* were characterized in this study. The ECPs of the four 2010 isolates were found to be toxic to channel catfish fingerlings, with LD_{50} value of 16 μg/fish. Thirty ECP fractions were obtained from the ECPs of the 2010 isolates of *A. hydrophila* by cation-exchange chromatography, of which nine fractions were found to be toxic to catfish gill cells and channel catfish fingerlings. Mass spectrometry identified 228 proteins from the nine toxic fractions, of which 23 were shared by toxic fractions, including well known virulence factors such as hemolysin, aerolysin, elastase (metalloprotease), nuclease, and 5′-nucleotidase. Hemolytic activity, protease activity, and nuclease activity of the four isolates were found to be significantly ($P < 0.05$) higher than that of a reference *A. hydrophila* strain AL98-C1B. Our results might shed light on the possible virulence factors of the highly virulent West Alabama isolates of *A. hydrophila*.

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1. Introduction

In West Alabama, disease outbreaks caused by *Aeromonas hydrophila* in 2009 have led to an estimated loss of more than \$3 million (Pridgeon and Klesius, 2011a,b). In 2010, MAS disease outbreak occurred again in West Alabama, causing losses of hundreds of thousands of pounds of market size channel catfish. As a gram-negative, motile, rod-shaped bacterium commonly found in aquatic environments throughout the world, *A. hydrophila* is the causative agent of motile aeromonad septicemia (MAS) (Harikrishnan et al., 2003), which is also known as epizootic ulcerative syndrome (Mastan and Qureshi, 2001). The symptoms of MAS include swelling of tissues,

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dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia (Karunasagar et al., 1989). Fish species affected by MAS include tilapia, catfish, goldfish, carp, and eel (Pridgeon and Klesius, 2012). The pathogenesis of *A. hydrophila* in fish involves multiple virulence factors. The extracellular products (ECPs) secreted by *A. hydrophila* are considered as essential virulent factors as they contain various proteins that possess cytotoxic, cytolytic, haemolytic, and enterotoxic properties, such as haemolysin (Allan and Stevenson, 1981) and protease (Yu et al., 2006).

During the 2010 MAS disease outbreak, four *A. hydrophila* isolates were obtained from kidney tissues of diseased catfish. However, it was unknown whether they were virulent to channel catfish. In addition, virulence factors of the 2010 West Alabama isolates of *A. hydrophila* have not been reported yet. Therefore, the objectives of this study are to: (1) perform biochemical and molecular identification for the four 2010 West Alabama isolates; (2) determine whether the 2010 West Alabama isolates were virulent to channel catfish; (3) determine whether the extracellular products (ECPs) of the 2010 West Alabama isolates were toxic to channel catfish; (4) identify proteins in toxic ECP fractions of the 2010 isolates by mass spectrometry.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

Four bacterial isolates (ML-10-51K, ML-10-81K, ML-10-205K, and ML-10-208K) were cultured from kidneys of diseased channel catfish from West Alabama fish farms during the 2010 disease outbreak (collected by Bill Hemstreet at the Alabama Fish Farming Center, Greensboro, AL). For relative virulence studies, the AL98-C1B isolate of *A. hydrophila* used for virulence studies of the 2009 West Alabama isolates of *A. hydrophila* (Pridgeon and Klesius, 2011a) was included as the reference strain for this study. All bacteria were inoculated onto tryptic soy agar plates and incubated at 28 °C for 16 h to 18 h. Bacterial cultures were then subjected to biochemical and molecular identification. A glycerol stock (10% glycerol) for all isolates was prepared in tryptic soy broth and stored at −80 °C.

2.2. Gram staining, oxidase test, catalase test, and API 20 NE test

Gram staining was performed using Gram staining kit (Beckton Dickinson, Franklin Lakes, NJ) following manufacturer's instruction. Oxidase test was performed by adding bacterial smear to filter paper containing Bacti-Drop oxidase reagent (Remel, Lenexa, KS). Color development was observed within 1 min. Catalase test was performed by adding one drop of 30% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) to a slide that contained bacterial smear. Bubbling reaction was observed within 1 min. API 20 NE bacterial identification was performed according to manufacturer's instruction (bioMérieux, Durham, NC).

2.3. Genomic DNA isolation and PCR

Genomic DNA was isolated from ML-10-51K using the DNeasy Kit (Qiagen, Valencia, CA). Published primers (Pridgeon and Klesius, 2011a) of *A. hydrophila* 16S–23S rDNA intergenic spacer region (ISR) were used in PCR. Purified PCR products were sent to USDA-ARS Mid-South Aera Genomic Laboratory (Stoneville, MS) for sequencing with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.4. Virulence of the 2010 isolates to channel catfish by intraperitoneal injection

Virulence assays were performed using published procedures (Pridgeon and Klesius, 2011a). Briefly, the four 2010 isolates and AL-98-C1B were cultured overnight in tryptic soy broth at 28 °C. After overnight incubation at 28 °C, the average number of bacteria was calculated for each isolate. The amount (colony forming units) of *A. hydrophila* used in this study was determined through serial dilutions. Briefly, an optical density (OD) of 1.0 of the bacterial cultures was measured at 540 nm using Thermo-spectronic spectrophotomer (Fisher Scientific, Pittsburgh, PA). Serial dilutions (in triplicates) of each *A. hydrophila* isolate were prepared in TSB and 100 μL of each dilution was plated onto TSA plates. After 24 h incubation at 28 °C, the average number of CFU/ml was calculated for each isolate. Channel catfish (30 ± 2 g) naïve to *A. hydrophila* exposure were randomly obtained from stocks maintained at the USDA-ARS-Aquatic Animal Health Research Unit at Auburn, AL and acclimated in individual experiment tanks for 14 days prior to challenge. The health status of catfish stock was evaluated by culturing kidney and brain tissues of randomly selected catfish on tryptic soy agar plates, and only culture negative fish stock was used in this study. For each isolate, six doses were injected to catfish. For each dose, 10 fish were used in each tank with three replicates. The experiments were repeated three times. Catfish were exposed to *A. hydrophila* by intraperitoneal injection at eight doses (for the 2010 isolates, the following eight doses were used: 3.2×10^6, 1.6×10^6, 8×10^5, 4×10^5, 2×10^5, 1×10^5, 5×10^4, 2.5×10^4 CFU/fish; for AL-98-C1B, the following eight doses were used: 8×10^8, 4×10^8, 2×10^8, 1×10^8, 5×10^7, 2.5×10^7, 1.25×10^7, 6.25×10^6 CFU/fish). Mortalities were recorded daily for 14 days post exposure. The presence or absence of *A. hydrophila* in dead fish was determined by culturing anterior kidney samples on blood agar plates followed by biochemical analysis. LD_{50} values were calculated using PoloPlus probit and logit analysis software (LeOra Software, Petaluma, CA). Virulence between different isolates was considered significantly different when the 95% confidence intervals of LD_{50} values failed to overlap ($P \leq 0.05$).

2.5. Preparation of extracellular products

Extracellular products were precipitated with 35% ammonium sulfate according to published procedures (Sirirat et al., 1999) with modifications. Briefly, *A. hydrophila* was grown in 100 ml TSB at 28 °C for

16–18 h. The bacterial culture was then centrifuged at $10,000 \times g$ for 30 min to separate supernatant from bacterial cells. ECPs were precipitated from the supernatant with 35% ammonium sulfate and dissolved in 2 ml of cold phosphate buffered saline (PBS, pH 7.2) in the presence of proteinase inhibitors (cOmplete Mini, EDTA free, Roche Diagnostics, Indianapolis, IN). The 2 ml ECPs were then dialysed against cold phosphate buffered saline (PBS, pH 7.2) for 1 h. The dialysis procedure was then repeated twice. Aliquot (2 ml) of ECPs were stored at −80 °C until needed. Concentration of ECPs was determined using a micro BCA protein assay kit (Pierce, Rockford, IL). Freshly prepared ECPs were used to inject fish immediately to determine its toxicity. For each fractions, six doses were injected to catfish. For each dose, 10 fish were used in each tank with three replicates. The experiments were repeated three times.

2.6. Library construction and personal genome machine sequencing

Personal genome machine (PGM) is a next-generation semiconductor sequencing platform that utilizes a small chip for detection of released hydrogen ions emitted during DNA polymerization (Rothberg and Myers, 2011). PGM has been successfully used to do fast identification for pathogens (Mellmann et al., 2011). Based on the fact that the four 2010 isolates shared similar virulence to channel catfish and that their 16S–23S sequences shared 100% identities with each other, only one isolate ML-10-51 K was chosen for PGM sequencing. Genomic DNA quality and concentration were determined by Agilent BioAnalyzer DNA 1000 LabChip (Agilent Technologies, Santa Clara, CA). Genomic DNA library was constructed using Ion Plus Fragment Library Kit (Life Technologies, San Francisco, CA) according to manufacturer's instructions. Data from the personal genome machine runs were processed initially using the Ion Torrent platform-specific pipeline software Torrent Suite v1.3.1 to generate sequence reads, trim adapter sequences, filter, and remove poor signal-profile reads. All sequences were analyzed using the National Center for Biotechnology Information (NCBI) BlastN program to search for sequence homologies.

2.7. Cation exchange chromatography separation of ML-10-51K ECPs

Since AL98-C1B ECPs failed to kill any fish at dose of 50 μg/fish whereas ECP of the four 2010 isolates killed 100% at similar dose (50 μg/fish), only ECP of the 2010 isolates were subjected to cation exchange chromatography. ECPs were loaded onto HiTrap™ CM FF 5 ml column (GE Healthcare Life-Sciences) connected to Biologic LP Chromatography system (Bio-Rad). After the column was equilibrated with 25 mM 2-N-morpholino-ethanesulfonic acid (MES) buffer (pH 6.2), bound material was eluted with a linear gradient (0–100%) of 1 M NaCl at a flow rate of 1.5 ml/min. The elution was monitored at 280 nm by a UV monitor provided by the Biologic LP Chromatography system and 2 ml of fraction was automatically collected. Each fraction was then precipitated with 35% ammonium sulfate, resuspended in PBS in the presence of proteinase inhibitors, and dialyzed against PBS. The concentrations of fractions were determined using BCA protein assay kit (Pierce, Rockford, IL).

2.8. Effect of ECP fractions on catfish gill cell's proliferation

Effect of ECP fractions on catfish gill cell's proliferation was performed according to published procedures (Pridgeon et al., 2011) with slight modifications. Briefly, catfish G1B gill cells in F-12K media (American Type Culture Collection, Manassas, VA) were split into 96-well tissue culture plates with final concentration of 1×10^5 cells per well and grown at 25 °C for 24 h. The number of gill cells was calculated microscopically using a hemacytometer with trypan blue exclusion method. ECP fractions (5 μg and 1 μg) in 100 μl F-12K media were added to each well. For each treatment, three replicates were included in each assay. Experiments were repeated three times. Gill cells with PBS and proteinase inhibitors added were used as positive proliferation controls. Plates were incubated at 25 °C for 24 h. At 24 h post incubation, the cell proliferation of G1B cells was determined by CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI) using published procedures (Pridgeon et al., 2011).

2.9. Toxicity of ECPs and ECP fractions to channel catfish by intraperitoneal injection

To study the toxicity of ECPs to channel catfish, various amounts (6, 8, 21, 30, 37, and 50 μg/fish) of ECPs were administrated to channel catfish fingerlings (10 fish/tank, triplicates) by intraperitoneal injection. To differentiate the toxicities of ECP fractions, the LD_{50} dose (16 μg per fish) was used to inject fish intraperitoneally. Channel catfish fingerlings (2 ± 0.5 g) naïve to *A. hydrophila* exposure were randomly obtained from stocks maintained at the USDA-ARS-Aquatic Animal Health Research Unit at Auburn, AL and acclimated for 14 days prior to challenge. Acclimated fish were maintained in 57 L glass aquaria with flow-through (0.5 l/min) de-chlorinated tap water and constant aeration with water temperature at 28 °C. After exposing catfish to the ECPs by intraperitoneal injection, mortalities were recorded daily for 14 days post exposure. For each fraction, a single dose (16 μg per fish) was used to inject catfish (10 fish per tank, 3 replicates per fraction). The experiments were repeated three times.

2.10. LC–MS/MS

Toxic ECP fractions were digested with trypsin (100 μg sequence grade trypsin per 1 mg of proteins) in 100 μl of Tris–HCl buffer, pH 8.0, at 37 °C for 2 h. Peptide digests were injected onto a Surveyor HPLC plus (Thermo Scientific, San Jose, CA) using a split flow configuration on the back end of a 100 μm I.D. × 13 cm pulled tip C-18 column (Jupiter C-18 300 Å, 5 μm, Phenomenex) using published procedures (Kojima et al., 2012). The MS/MS scans were obtained in normal mode with a minimum signal threshold of 500 counts based on the SIM scan.

Searches were performed with an *A. hydrophila* subset of the UniRef100 database, which included common contaminants such as digestion enzymes and human keratin. A list of protein IDs was generated based on SEQUEST search results, which were filtered based on calculated peptide and protein probabilities, followed by grouping of top scoring protein IDs with Scaffold (v.3, Proteome Software, Portland, OR).

2.11. Hemolytic activity, protease activity, and nuclease activity assays

The determination of hemolytic activity of the 2010 isolates compared to that of AL98-C1B strain was performed using published procedures (Singh et al., 2010) with slight modifications. Briefly, similar amount of the 2010 isolates or AL98-C1B was inoculated onto 5% sheep blood agar plates. The determination of protease activity was performed using published procedures (Ribitsch et al., 2012). Briefly, similar amount of the 2010 isolates or AL98-C1B was inoculated onto tryptic soy agar plates containing 1% skim milk. The amount (colony forming units) of *A. hydrophila* used in this study was determined through serial dilutions. Briefly, an optical density (OD) of 1.0 of the bacterial cultures was measured at 540 nm using Thermospectronic spectrophotomer (Fisher Scientific, Pittsburgh, PA). Serial dilutions (in triplicates) of each *A. hydrophila* isolate were prepared in TSB and 100 μL of each dilution was plated onto TSA plates. After 24 h incubation at 28 °C, the average number of CFU/ml was calculated for each isolate. The determination of nuclease activity was performed using published procedures (Sinicropi et al., 1994) with slight modification. Briefly, similar amount of bacteria of the 2010 isolates and that of AL98-C1B were inoculated onto agar plates containing 0.2% deoxyribonucleic acid and 0.005% methyl green (Difco^{TM} DNase Test Agar with Methyl Green, Becton Dickinson, Franklin Lakes, NJ). After incubating the plates at 28 °C for 48 h, the zone clearance surrounding the inoculation area was photographed and the diameter of the clear zone was measured.

2.12. Statistical analysis

All statistical analyses were performed using SigmaStat 3.5 software (Systat Software, Inc., Point Richmond, CA). Differences in virulence, hemolytic activity, protease activity, and nuclease activity were analyzed with student *t*-test and the significance level was defined as $P < 0.05$.

3. Results

3.1. Gram staining, oxidase test, catalase test, and API 20 NE test

Gram staining, oxidase tests and catalase tests revealed the four 2010 isolates were Gram-negative, oxidase-positive, and catalase-positive. API 20 E results revealed that the 7-digit numerical profile for the four 2010 isolates were 7-0-0-6-1-2-6, which was identified as *A. hydrophila* with 97.6% identification.

3.2. 16S–23S ISR sequencing results

BLAST sequence analysis revealed that the 16S–23S ISR of the four 2010 isolates shared 100% identities with each other. The 16S–23S ISR of the four 2010 isolates shared the highest sequence identities (86%) with the three 2009 West Alabama isolates of *A. hydrophila*: AL09-71 (accession no. HM856361), AL09-72 (accession no. HM856359), and AL09-73 (accession no. HM856360), with e-values = 7e − 91, 6e − 88, and 2e − 87, respectively. The 16S–23S ISR sequences of the four 2010 isolates also shared 81% identities with *A. hydrophila* ATCC 21763 (accession no. HQ389224) and ATCC 7966 (accession no. CP000462), with e-value = 5e − 9. The 16S–23S ISR sequence of the 2010 isolate ML-10-51K was deposited at GenBank under accession number KC511816.

3.3. PGM sequencing results

Raw PGM data of ML-10-51K genomic DNA were ~1 M bp. BlastN sequence analysis revealed that the ML-10-51K genomic DNA had the highest query coverage with *A. hydrophila subsp. hydrophila* ATCC 7966 complete genome (accession no. CP000462). The genomic DNA sequences of ML-10-51K obtained from PGM run contained sequences of 128 unique *A. hydrophila* genes. The 128 sequences were deposited at GenBank under accession number JY253262–JY253390. Representative ML-10-51K genes that shared identities with *A. hydrophila* ATCC 7966 are listed in Supplementary Table 1, including partial gene sequences such as ribosomal protein S6 and NlpB/DapX lipoprotein that shared 100% identity match with *A. hydrophila subsp. hydrophila* ATCC 7966 (accession no. CP000462) (Supplementary Table 1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.02.020.

3.4. Virulence of the 2010 isolates to channel catfish by intraperitoneal injection

The LD_{50} values of the 2010 isolates to channel catfish were determined to be 1.3×10^5 CFU/fish, with 95% confidence interval ranging from 5×10^4 to 2.9×10^5 CFU/fish. The LD_{50} value of AL98-C1B strain was 2.8×10^7 CFU/fish, with 95% confidence interval ranging from 1.2×10^7 to 4.5×10^7 CFU/fish. Based on LD_{50} values, the 2010 isolates were at least 200 times more virulent to channel catfish than the AL98-C1B strain.

3.5. Toxicity of ECPs to channel catfish fingerlings by intraperitoneal injection

The ECPs of the 2010 isolates were found to be toxic to channel catfish fingerlings, killing 10%, 20%, 50%, 90%, 100%, and 100% channel catfish fingerlings at doses of 6, 8, 21, 30, 37, and 50 μg/fish, respectively. However, at all doses tested (6, 8, 21, 30, 37, and 50 μg/fish), ECPs of AL98-C1B failed to kill any fish. The LD_{50} value of ML-10-51K ECP to channel catfish fingerlings was determined to be 16 μg/fish, with 95% confidence interval ranging from 11 to 25 μg/fish.

3.6. Cation-exchange chromatography and toxicity of fractions

A total of 30 fractions were collected from the ECPs of the 2010 isolates by cation-exchange chromatography (Supplementary Fig. 1). Of the 30 fractions, 21 (fraction 7–26) contained measurable proteins. Of the 21 fractions, 9 (fraction 12–20) significantly ($P < 0.05$) inhibited the proliferation of catfish gill cells compared to other fractions when 5 μg of proteins were added to each well (Fig. 1A) or 1 μg of ECP proteins were added to each well (Fig. 1B). When 16 μg of ECP fractions were injected to channel catfish fingerlings, fractions 12–20 killed 30%–60% fish, whereas other fractions failed to kill any fish. Dead fish after injection of ECP fractions showed obvious symptom of hemorrhagic septicemia (Supplementary Fig. 2A), which is similar to the dead fish after injection of total ECP (Supplementary Fig. 2B).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.02.020.

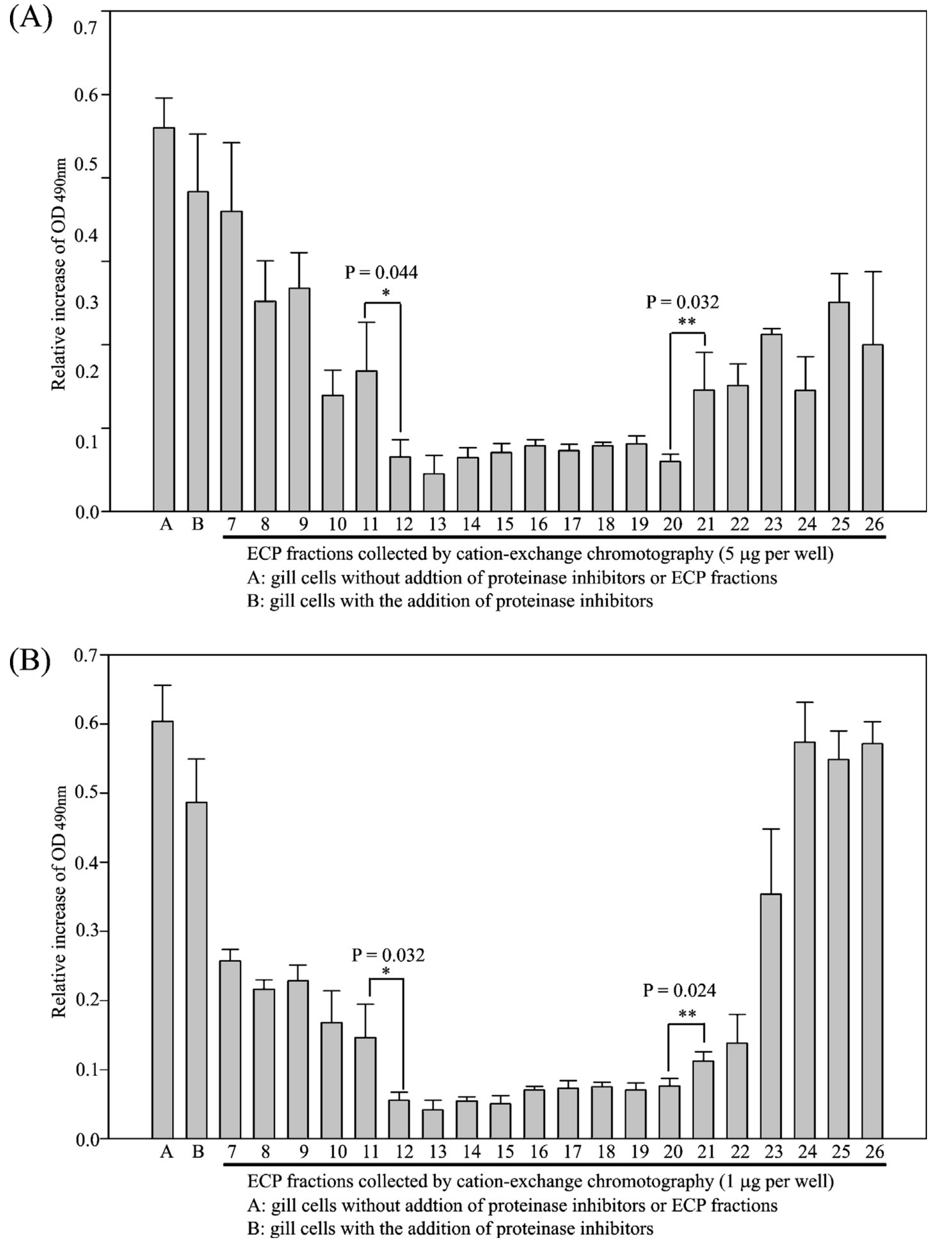

Fig. 1. Effect of *A. hydrophila* ML-10-51K ECP fractions on the cell proliferation of catfish gill cell G1B. (A) Effect of 5 μg of ECP fractions on the cell proliferation of G1B; (B) Effect of 1 μg of ECP fractions on the cell proliferation of G1B. Cell proliferation of G1B cells was determined by CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay. Relative increased OD 490 nm values were calculated using the following formula: Increased OD value = OD value after incubation − OD value at 0 h of the incubation. Data were presented as mean ± standard deviation (S.D.) from three replicates. Significant difference ($P < 0.05$) was marked by asterisk.

Table 1
List of the 23 proteins shared by the nine toxic ECP fractions.

Identified protein	Accession number	MW (kDa)	Putative function
6,7-Dimethyl-8-ribityllumazine synthase	YP_857818	21	Biosynthesis
Arginine deiminase	YP_858517	46	Metabolism
Glyceraldehyde-3-phosphate dehydrogenase	YP_858088	35	Metabolism
Methionine gamma-lyase	YP_856478	41	Metabolism
Ornithine carbamoyltransferase	YP_858515	38	Metabolism
Putative uncharacterized protein	YP_855640	23	Unknown
Putative uncharacterized protein	YP_857944	16	Unknown
Putative uncharacterized protein	YP_858108	23	Unknown
Putative uncharacterized protein	YP_858275	78	Unknown
5′-Nucleotidase/2′,3′-cyclic phosphodiesterase	YP_855554	66	Virulence
Aerolysin	ABR14714	51	Virulence
Chitin binding protein	YP_855149	54	Virulence
Elastase	YP_855393	63	Virulence
FlaA	ABG56543	32	Virulence
Flagellar hook protein	ADQ26724	47	Virulence
Hemolysin ahh1	YP_856050	69	Virulence
Lipase	F1SW09	83	Virulence
Long-chain fatty acid transport protein	YP_856669	47	Virulence
Major adhesin Aha1	ABC54617	39	Virulence
Maltoporin	A0KHF6	48	Virulence
Nuclease	YP_857930	77	Virulence
Outer membrane porin protein	YP_855396	39	Virulence
Outer membrane protein	YP_855671	32	Virulence

3.7. LC–MS/MS results of toxic ECP fractions

A total of 228 proteins were identified from the toxic ECP fractions of ML-10-51K by LC–MS/MS (supplementary Table 2). Of the 228 proteins, 23 were identified in all replicates of the nine toxic fractions. The accession number, molecular weight and putative function of the 23 proteins shared by the nine toxic ECP fractions are summarized in Table 2. Based on putative functions, majority (14/23 = 61%) of the 23 proteins are related to virulence, including well known virulence factors such as hemolysin, elastase (protease), and nuclease (Table 1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.02.020.

3.8. Hemolytic activity, protease activity, and nuclease activity

Hemolytic activities of the 2010 isolates were found to be significantly ($P < 0.05$) higher than that of AL98-C1B. Similarly, the protease and the nuclease activities of the 2010 isolates ML-10-51 K were significantly ($P < 0.05$) higher than that of AL98-C1B in that the zone around the ML-10-51K inoculation area was significantly ($P < 0.05$) larger than that around the AL98-C1B inoculation area.

4. Discussion

Precise identification of pathogen is essential to successful control of disease outbreak. Identification of *Aeromonas* at the species level by routine procedures involves many difficulties because of the absence of a unified identification key and the lack of agreement between biochemical and genetic identification schemes (Soler et al., 2004; Ormen et al., 2005). Sequencing of the 16S–23S rDNA intergenic spacer region is now considered a robust and sensitive taxonomic tool which is widely used in bacterial taxonomy (Martínez-Murcia et al., 2005; Tazumi et al., 2009). Sequencing results of the 16S–23S ISR of the 2010 West Alabama isolates revealed that it shared highest identities with the three 2009 West Alabama isolates of *A. hydrophila*, confirming that 16S–23S ISR sequencing is a useful tool in bacterial taxonomy. The identities between the 2010 West Alabama isolate ML-10-51K and the three 2009 isolates were only 86%, whereas the identities among the ISRs of the four 2010 isolates were 100%. The LD_{50} value of the 2010 West Alabama isolates to 30 g channel catfish was determined to be 1.3×10^5 CFU/fish, which is similar to the LD_{50} values (1.1×10^5–1.9×10^5 CFU/fish) of the three 2009 West Alabama isolates of *A. hydrophila* to 30 g channel catfish that was reported previously (Pridgeon and Klesius, 2011a). These results suggested that the virulence of the 2010 isolates were not significantly different from that of the 2009 isolates, although the ISR sequences of the 2010 isolate differed from that of the 2009 isolates. Both analytical profile index (API) 20 E biochemical test and Ion Torrent PGM sequencing confirmed the identities of the 2010 isolates as *A. hydrophila*. PGM is a next-generation semiconductor sequencing platform that utilizes a small chip for detection of released hydrogen ions emitted during DNA polymerization (Rothberg and Myers, 2011). PGM has successfully identified the exceptionally virulent Shiga toxin (Stx)-producing *Escherichia coli* O104:H4 centered in Germany, which has caused over 830 cases of hemolytic uremic syndrome (HUS) and 46 deaths since May 2011 (Mellmann et al., 2011). It was reported that draft assembly of the strain was completed with the newly introduced PGM™ machine within 62 h (Mellmann et al., 2011). We finished the library construction and PGM running within one week, suggesting that PGM could be used to do fast identification of microorganisms. However, this technology is much more expensive than traditional molecular sequencing for the purpose of bacterial identification. In addition, PGM is known for its inaccuracy of homopolymers (Yeo et al., 2012), which we also observed in this study (the discrepancy between our sequences and the sequences deposited at GenBank was those homopolymers), resulting in less than 100% identities between query and subject. Nonetheless, PGM can be used to do fast identification of microorganisms, especially if sequence information is sparse or unknown for an organism.

The pathogenesis of *A. hydrophila* in fish involves multiple virulence factors. The extracellular products (ECPs) secreted by *A. hydrophila* are considered as essential virulent factors as they contain various proteins that possess cytotoxic, cytolytic, haemolytic, and enterotoxic properties. The ECPs of the 2010 isolates were found to be toxic to channel catfish fingerlings, with LD_{50} value of 16 μg/fish (95% confidence interval ranging from 11 to 25 μg/fish). In addition, nine ECP fractions collected by cation-exchange chromatography were found to be toxic to catfish gill cells and channel catfish fingerlings. Toxicity of ECPs of *A. hydrophila* to fish such as tilapia has been reported previously (Khalil and Mansour, 1997). Mass spectrometry identified 228 proteins from the nine toxic fractions, of which 23 were shared by the nine toxic fractions, including well known virulence factors such as hemolysin and aerolysin. It was reported that the inactivation of hemolysin and aerolysin by mutagenesis attenuated the virulence of *A. hydrophila* (Wong et al., 1998). The hemolytic activities of the 2010 isolates ML-10-51K were found to be higher than that of AL98-C1B. This finding is not surprising since hemolytic activity has been suggested as a significant lethality factor of *A. hydrophila* to fish (Allan and Stevenson, 1981). Taken together, these results suggest that hemolysin and aerolysin might play critical role in the virulence of the 2010 West Alabama isolates of *A. hydrophila*. LC–MS/MS also identified elastase (metalloprotease) in all nine toxic ECP fractions. The 63 kDa elastase has been demonstrated to be essential for the pathogenicity of *A. hydrophila* (Cascón et al., 2000). Based on LD_{50} values, the virulence of the elastase mutant of *A. hydrophila* to rainbow trout (Oncorhynchus mykiss) was 50 times less than the wild type strain of *A. hydrophila* (LD_{50} for the wild-type strain was 6×10^5 CFU/fish whereas the LD_{50} for the elastase mutant was 3×10^7 CFU) (Cascón et al., 2000). In addition, elastase was reported to be able to activate aerolysin (Song et al., 2004). In this study, we found that the protease activity of the 2010 isolates was significantly higher than that of AL98-C1B. Taken together, these results suggest that elastase might be essential for the virulence of the 2010 West Alabama isolates of *A. hydrophila*. In addition to hemolysin, aerolysin, and elastase mentioned above, nuclease and 5′-nucleotidase were also identified in all nine toxic ECP fractions by LC–MS/MS. Although the functions of nuclease and nucleotidase in the virulence of *A. hydrophila* are currently unknown, their functions as virulence factors have been reported in other bacteria. A knockout mutant of *Streptococcus pyogenes* nuclease *SpnA* was reported to be less virulent in blood bactericidal assays and in a mouse infection model (Hasegawa et al., 2010). A deletion mutant of 5′-nucleotidase was reported to have significantly shorter lag time to onset of platelet aggregation than the wild-type strain of *Streptococcus sanguinis*, the most common cause of infective endocarditis (Fan et al., 2012). In this study, we found that the nuclease activity of the highly virulent ML-10-51K appeared to be higher than that of the less virulent AL98-C1B. Taken together, these results suggest that nuclease and nucleotidase might also be important virulence factors of the 2010 West Alabama isolates of *A. hydrophila*. Besides the five proteins discussed above, 18 more proteins were present in all nine toxic ECP fractions, including some functionally unknown proteins. Furthermore, some proteins such as extracellular protease, extracellular serine protease, and flagellar hook-associated FlgK were identified in 17 of the 18 toxic ECP fraction samples, suggesting that multiple virulence factors have contributed to the lethality of the 2010 West Alabama isolates of *A. hydrophila* to channel catfish.

In summary, both analytical profile index (API) 20 E biochemical tests and Ion Torrent personal genome machine sequencing confirmed the identity of the 2010 West Alabama isolates as *A. hydrophila*. Virulence studies revealed that the 2010 West Alabama isolates of *A. hydrophila* were highly virulent to channel catfish by intraperitoneal (IP) injection. The ECPs of the 2010 West Alabama isolates of *A. hydrophila* were also found to be toxic to channel catfish fingerlings. Thirty ECP fractions were obtained from cation-exchange chromatography, of which nine were found to be toxic to catfish gill cells and channel catfish fingerlings. Mass spectrometry identified 228 proteins from the nine toxic fractions, of which 23 were shared by the nine toxic fractions, including well known virulence factors such as hemolysin, aerolysin, elastase (metalloprotease), nuclease, and 5′-nucleotidase. Hemolytic activity, protease activity, and nuclease activity of the 2010 West Alabama isolates of *A. hydrophila* were found to be higher than that of a reference *A. hydrophila* strain AL98-C1B. Our results might shed light on the possible virulence factors of the highly virulent 2010 West Alabama isolates of *A. hydrophila*.

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